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Oral administration of *N*-acetyl Glucosamine alters the mRNA expression profile of hyaluronan-related genes in mouse skin

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This study conducted *in vitro* and *in vivo* investigation of the impact of D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc) intake on the skin of C57BL/6J mice. The results of *in vivo* investigation by DNA microarray analysis indicated that GlcNAc intake had increased the expression of hyaluronan (HA)-related genes, including hyaluronan synthase 2 (*HAS2*) and its receptor, a receptor for hyaluronan-mediated motility (*RHAMM*), in the mouse skin. The results of *in vitro* investigation using mouse fibroblasts by toluidine blue staining indicated that GlcNAc had increased glycosaminoglycan content, while the results of RT-PCR analysis indicated that GlcNAc had increased the mRNA expression levels of *HAS2* and *RHAMM*. These results suggest that intake of GlcNAc but not GlcN may increase the HA content in skin by increasing the mRNA expression levels of *HAS2* and *RHAMM*.

Keywords: *N*-acetyl glucosamine, dermal tissue, hyaluronan, hyaluronan synthase 2, glucosamine

1. Introduction

D-Glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc) are precursors of glycosaminoglycans (GAGs), including hyaluronan (HA), produced through the hexosamine biosynthesis pathway in the body[1]. The structure of HA is that of a linear polysaccharide composed of repeating disaccharide units of glucuronic acid (GlcA) (β1→3) GlcNAc (β1→4). Typically, the full-length polymer of HA has a very high molecular mass, ranging from 10^5 kDa to 10^7 kDa, which allows it to serve as a space filler, an osmotic buffer, and a viscoelastic structure[2].

HA is a major component of connective tissue, with the largest amount residing in cartilaginous and dermal tissues, where it assumes an important role in cartilage and skin hydration and elasticity[3, 4]. The importance of its role can be observed with the age-related changes (e.g., wrinkling) that occur with a decline in HA content with aging[5]. As such, the addition of HA in some form to cosmetics and wound healing preparations and materials has been proposed. However, because little is known about the digestion and absorption of orally ingested HA, as well as of all high molecular mass compounds, its potentially beneficial effects cannot be predicted adequately.

Because of their chemical structure, namely that of a monosaccharide HA precursor, GlcN and GlcNAc are more easily absorbed in the small intestine when administered orally or applied topically than are more complex polysaccharides, including HA[6, 7]. As such, direct application of

GlcN and GlcNAc to the skin may provide several cosmetic and health benefits, such as enhanced skin hydration and promotion of wound healing. One study found that oral GlcNAc supplementation may enhance skin hydration and decrease skin roughness[8]. Nevertheless, very little sound scientific research has been conducted into the benefits of GlcN and GlcNAc supplementation for skin hydration and wound healing. As such, the mechanism by which GlcN and GlcNAc provides these benefits, if they indeed do so, is still not well understood, and their effectiveness remains controversial.

Recent research has found that GlcN and GlcNAc affect intracellular signaling and regulate the mRNA expression of chondrocyte- or osteoblast-specific genes[9-11]. Based on this finding, it has been hypothesized that GlcN and GlcNAc alter gene expression in dermal tissue in a manner that promotes skin hydration and wound healing. However, the research upon which this hypothesis is based did not examine whether the manner in which GlcN and GlcNAc is administered (i.e., orally or topically) affects gene expression in dermal tissue. To fill these research gaps, this study used Codelink Microarray technology (VERITAS Corporation, Tokyo, Japan) for gene expression profiling of mouse dermal tissue treated with GlcN and GlcNAc. Such analysis allowed for identification of whether oral administration of glucose supplemented with GlcN or GlcNAc resulted in significant differences in the up-regulation or down-regulation of differentially expressed genes compared to oral administration of glucose only, as well as determination of whether GlcN and GlcNAc supplementation affected genes in a different manner. Using KeyMolnet software, DNA microarray was performed and genetic databases were reviewed to determine the manner in which GlcNAc administration changed the molecular relationships among the genes and the results verified by experimentation with a mouse fibroblast. Finally, the molecular mechanism by which oral GlcN or GlcNAc supplementation increases the moisture content of mouse dermal tissue was identified.

2. Experiment

2.1 Animals and diets

Male C57BL/6 mice were obtained from Clea Japan (Tokyo, Japan) at 9 wk of age and fed a normal laboratory diet for 1 wk to achieve metabolic stabilization. The mice were housed under conditions of constant temperature (20°C to 22°C) and humidity (45% to 50%) according to a standard 12-h light/dark cycle. To perform DNA microarray analysis, the mice were randomly divided into 3 groups of 6 mice each, one of which was administered a 0.02% glucose-containing diet (the control group), one a 0.02% GlcN×HCl-containing diet, and one a 0.02% GlcNAc-containing diet for 3 weeks. Glucose, GlcN×HCl and GlcNAc were purchased from Sigma-Aldrich Japan (Tokyo, Japan). All diets used the *in vivo* experiments were modifications of the AIN-93G composition, with glucose, GlcN×HCl, or GlcNAc replacing the relevant amount of cornstarch in the diet. At the end of the study period, the animals were anesthetized by intraperitoneal injection of pentobarbital sodium (0.08 mg/g body wt; Kyoritsu Seiyaku Corporation, Tokyo, Japan). After the abdominal hair had been shaven, the abdominal skin and liver were isolated and immediately homogenized with Trizol Reagent (Invitrogen, Tokyo, Japan).

The study was performed in accordance with National Institute of Health (NIH; Bethesda, MD, USA) guidelines for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Institutional Laboratory Animal Care and Use Committee of the University of Josai, Japan.

2.2 RNA extraction

Total RNA was isolated using Trizol Reagent and purified using the RNeasy Mini Kit (Qiagen, Duesseldorf, Germany) according to the

manufacturer's instructions. The quality of the RNA samples was verified by spectrophotometry and agarose gel electrophoresis. RNA samples were used for cRNA target preparation only when the ratio A_{260}:A_{280} was 1.8:2.1. The RNA was stored at -80°C prior to processing for DNA microarray and reverse transcription polymerase chain reaction (RT-PCR) analysis.

2.3 DNA microarray

DNA microarray was performed on the 3 treatment groups by pooling an equal amount of RNA taken from each mouse within each group for microarray analysis. The CodeLink Expression Bioarray System (VERITAS Corporation, Tokyo, Japan) was used for cRNA target preparation and bioarray hybridization detection. Following assessment of total RNA concentration and quality, 1 μg total of the RNA sample from each group and bacterial control mRNA were incubated with T7 olido (dT) primer for 10 min at 70°C. Synthesis of first-strand and second-strand cDNA was performed at 42°C for 2 h and at 16°C for 2 h, respectively. Using an *in vitro* transcription (IVT) mix containing biotinylated UTP, ribonucleotides, 10 × T7 enzyme mix, and cDNA, double-stranded cDNA that had been purified using the QIAquick PCR Purification Kit (Qiagen, Duesseldorf, Germany) was used for synthesis of cRNA by IVT at 37°C for 14 h. The resulting purified biotin-labeled cRNA was assessed for concentration, purity, and quantity. Codelink bioarrays were processed only when the ratio A_{260}:A_{280} was 1.8:2.1.

Using a series of CodeLink Mouse Whole Genome Bioarrays, 10 μg of RNA was fragmented at 94°C for 20 min with the fragmentation buffer per bioarray. The fragmented cRNA was incubated at 90°C for 5 min with a hybridization mixture (hybridization buffer components A and B) before being chilled on ice for 5 to 30 min. A reaction mixture (250 μl) for each microarray was loaded into array chambers and hybridized at 37°C in a shaker (300 rpm) for 18 to 24 h, maintaining a consistent hybridization time for comparative experiments. Streptavidin-dye conjugate was used for detection. The bioarrays were washed with 0.75 × TNT at 46°C for 1 h and incubated with Cy5-Streptavidin for 30 min at room temperature. Following 4 washes of 5 min each with 1 × TNT (0.10 M of Tris HCl, pH 7.6; 0.15 M of NaCl; and 0.05% Tween-20), the bioarrays were rinsed with 0.1 × SSC (0.015 M NaCl, 1.5 mM Sodium Cirate) /0.05% Tween for 30 s. After drying, the bioarrays were immediately scanned with an Axon GenePix Scanner (Molecular Devices Japan KK, Tokyo, Japan) using the setting suggested by the supplier (VERITAS Corporation, Tokyo, Japan) for CodeLink bioarrays (resolution, 5 μm; laser wavelength, 635 nm; electric potential value of PMT, 600 V).

2.4 Data analysis

Initial data analysis was performed using CodeLink Expression Analysis v4.0 software (GE Healthcare Japan, Tokyo, Japan) to guarantee that high-quality data would be extracted from the CodeLink bioarray images generated from the scanner. The mean intensity obtained for each spot and background was corrected by subtracting the surrounding median local background intensity. Raw intensities were normalized using the global median for each bioarray before further analysis of the quantified data. The genes of GlcN×HCl- or GlcNAc-supplemented mice whose expression had increased or decreased more than 2-fold relative to the genes of the glucose-supplemented mice genes were identified and examined to determine the correlation between GlcN or GlcNAc supplementation and the expression of certain genes.

Gene annotation and pathway analysis were performed using KeyMolnet Lite (IMMD Inc., Tokyo, Japan), a knowledge-based content database manually curated by expert biologists that contains data regarding the relationships among human genes, molecules, diseases, pathways, and drugs. They are categorized into the core contents collected

from selected review articles with the highest reliability or the secondary contents extracted from the abstracts contained in the PubMed database. The "molecular relation pathway" is a network-search algorithm used to identify the molecular interaction network constructed by an upregulated gene set compared with a control gene set.

2.5 Cell culture

Dermal cells were prepared from the skin of newborn normal ICR mice by explant culture within 24 h of birth. The extracted skin was incubated for fragmentation by collagenase (Wako Pure Chemical Industries, Osaka, Japan) at 37°C for 30 min. The tissue fragments were grown at 37°C in 5% CO_2 in 100-mm tissue-culture-grade dishes in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and a penicillin (50 IU/mL, Meiji Co., Ltd., Tokyo, Japan)–streptomycin (50 μg/mL, Meiji Co., Ltd., Tokyo, Japan) solution until a sufficient number of cells had grown from the explant to establish a monolayer culture. On reaching confluency, the cells were trypsinized from the dish and passaged into 60-mm tissue-culture-grade dishes. The cells were passaged for 2 or 3 days to establish the fibroblasts as the predominant cells in the culture, and only used for implantation after 4 passages.

2.6 Cell proliferation assay

Dermal fibroblasts were cultured in 96-well plates with approximately 5×10^3 cells per well. After culturing for 24 h, the medium was replaced with an aliquot (100 μL) of a basic medium, a basic medium containing 5 mM GlcN · HCl, or a basic medium containing 5 mM GlcNAc. The cells were cultured for 2 days before the extent of cell-proliferation was determined by tetrazolium salt assay using WST-1 Cell Proliferation Assay Kit (Roche Diagnostics Japan, Tokyo, Japan). After culturing for 2 days, 10 μL of WST-1 reagent was added into each well and incubated for 4 h at 37°C, in accordance with the manufacturer's instructions. The 96-well plate was placed into a microplate reader, and the absorbance at 440 nm was measured for each well.

2.7 Toluidine blue staining

Dermal fibroblasts were cultured in 24-well plates with approximately 2×10^4 cells per well. After culturing for 24 hours, the medium was replaced with an aliquot (1 mL) of a basic medium, a basic medium containing 5 mM GlcN · HCl, or a basic medium containing 5 mM GlcNAc. The cells were cultured for 2 days before toluidine blue staining was performed to detect the presence of acid mucopolysaccharides around the extracellular matrix of the fibroblasts. After the cells had been fixed with 20% formalin, they were stained with 0.05% toluidine blue solution (pH 4.1; Muto Pure Chemicals, Tokyo, Japan) for 10 min. The number of areas of staining was counted under a light microscope. The extent of the toluidine-blue-stained area, which indicates GAG production, was quantified by densitometric analysis using videomicroscopy and ImageJ software (NIH, Bethesda, MD, USA) and the signals were normalized by basic medium cultured.

2.8 Reverse transcription polymerase chain reaction (RT-PCR)

cDNA was prepared from 1 μg of total RNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Japan). Amplification was performed in a 10 μL reaction mixturee containing 1 μL of cDNA reaction and using EX Taq (Takara Bio, Shiga, Japan). The primer sequences for *hyaluronan synthase 2* (*HAS2*) were 5′-TCTGGACATCTCCTCCAACAC-3′ (upstream) and 5′-AACGGTAGCACTCTGCATCG-3′ (downstream); for a receptor for hyaluronan-mediated motility (*RHAMM*) 5′-CCACTTGATCAGATGCACAGC-3′ (upstream); and for *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) 5′-TTGACCTCAACTACATGG-3′ (upstream) and 5′-CAGGGTGGTGGACCTCAT-3′ (downstream). Initial denaturation was performed at

94°C for 30 s, with annealing temperatures ranging from 56 to 58°C for 30 s, with an extension at 72°C and a final extension at 72°C for 3 min to conclude the reaction. The PCR products were resolved on a 1.5% agarose gel and visualized using ethidium bromide. All gels were digitally imaged using the Gel Doc EZ System (Bio-Rad Japan, Tokyo, Japan). Within any series, all adjustments were made in parallel to all gels used for comparison. Band intensities of the digital images were determined using Gel Doc EZ System software. The signals were normalized by those of GAPDH transcripts.

3. Results

3.1 Differential gene expression analysis

Of the 36,000 genes analyzed by gene expression profiling, 414 (1.15%) genes in the GlcN-supplemented mice were found to have been significantly and substantially upregulated and 584 (1.62%) to have been significantly downregulated compared with those in the control mice (**Fig. 1**). In the GlcNAc-supplemented mice, 2,415 (6.71%) genes were found to have been significantly upregulated and 1,038 (2.88%) to have been significantly downregulated compared with those of the control mice (**Fig. 1**). No differences were observed among the 3 groups regarding body weight and food consumption during the entire study period (data not shown).

3.2 Upregulated genes in GlcNAc-supplemented mice

To examine the relationships among the many genes upregulated in the GlcNAc-supplemented mice, gene cluster analysis was performed using the KeyMolnet Lite software, which provides a database of molecular interactions based on the findings reported in major scientific articles, focusing on the gene related to HA expression. Whereas *HAS2* (Mm. 5148) expression was found to have been upregulated by approximately 4.3-fold in the GlcNAc-supplemented mice and approximately

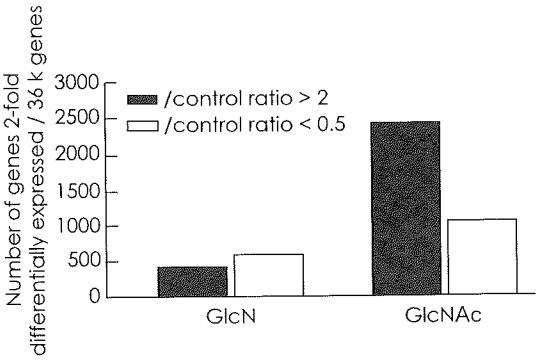

Fig. 1. Number of differentially expressed genes in mice after 3 weeks of GlcN or GlcNAc supplementation. Microarray analysis of genes differentially expressed by at least 2-fold revealed the distinct gene expression profiles of dermal tissue from the GlcN- and GlcNAc-supplemented mice compared to the control mice.

1.1-fold in the GlcN-supplemented mice compared to the control mice (Fig. 2), no differences in *HAS1* or *HAS3* expression were found among the 3 groups (data not shown). Analysis of HA expression using the KeyMolnet Lite software indicated the existence of an interaction among 8 genes related to HA metabolism or an HA pathway, which was further examined.

Degradation of HA is mediated by hyaluronidase (*HYAL*), an HA-degrading enzyme. As *HYAL2* primers are located to alleyed at 2 points (GE1531517, GE35579) in the CodeLink Mouse Whole Genome Bioarrays, *HYAL2* expression could be analyzed at the 2 points. The results of the analysis indicated that whereas *HYAL2* (NM_010489.2) expression in the GlcNAc-supplemented mice had been upregulated by approximately 2.6- and 3.0-fold at the 2 points compared to the control mice, *HYAL2* expression in the GlcN-supplemented had been upregulated by only approximately 0.6- and 1.6-fold. In contrast, no significant differences were found among the *HYAL1* and *HYAL3* expression levels of the 3 groups (data not shown).

The genes of several proteins associated with HA

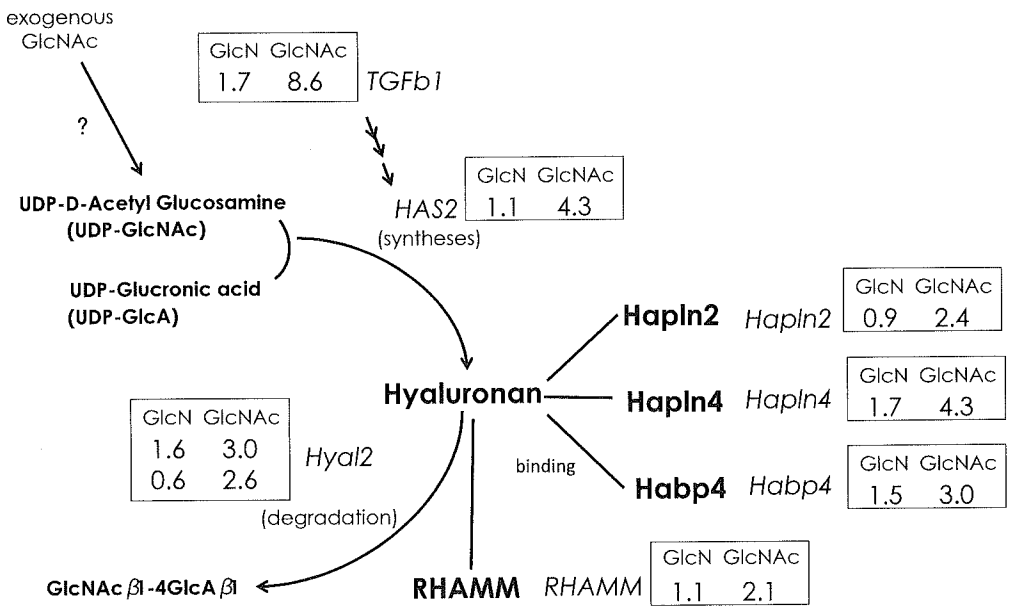

Fig. 2. Genes involved in HA biosynthesis exhibiting elevated expression in GlcN or GlcNAc supplemented mice. Numbers in boxes indicate the fold differences in gene expression compared to control mice. The dotted line indicates the binding of a saccharide and a protein or its receptor. The short dotted arrow indicates the origin of composition and the long dotted arrow indicates that the gene upregulated the down-stream other gene.

were also found to have been upregulated by GlcNAc supplementation. Specifically, the expression levels of 2 hyaluronan and proteoglycan link proteins (*Hapln),* namely *Hapln2* (NM_022031.1) and *Hapln4* (NM_177900.3), were found to have been upregulated by approximately 2.4- and 2.0-fold, respectively, in the GlcNAc-supplemented mice and by approximately 0.9- and 1.7-fold, respectively, in the GlcN-supplemented mice compared to the control mice (**Fig. 2**). In addition, the expression level of hyaluronic acid binding protein 4 (*HABP4*; NM_019986.1) was found to have been upregulated by approximately 3.0- and 1.5-fold in the GlcNAc- and GlcN-supplemented mice, respectively, compared to the control mice. In contrast, the expression level of *RHAMM* (Mm.116997) was found to have been upregulated by approximately 2.1-fold in the GlcNAc-supplemented mice (**Fig. 2**) but not to have been upregulated to any extent in the GlcN-supplemented mice (data not shown) compared to the control mice.

These findings indicate that expression levels of HA-associated genes had been affected by oral administration of GlcNAc than that of GlcN. As the next step in the analysis, KeyMolnet Lite software was used to identify the factor that induced increased HA expression. The results of the analysis indicate the involvement of transforming growth factor beta 1 (*TGFb1*; Mm.248380), whose expression level was found to have been upregulated by approximately 8.6-fold in the GlcNAc-supplemented mice compared to the control mice (**Fig. 2**).

3.3 Effect of GlcN and GlcNAc supplementation on GAG content and localization in the mouse fibroblast

The *in vivo* component of this study analyzed the gene expression of a mixture of skin tissues

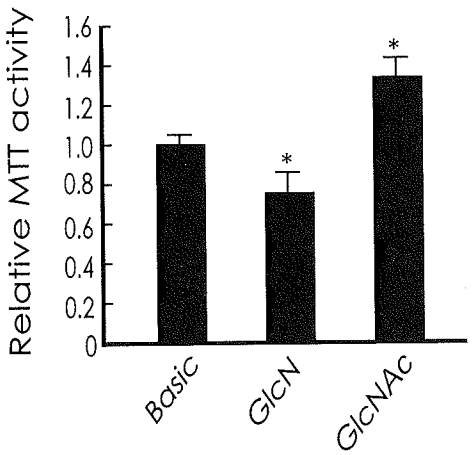

Fig. 3. Effect of GlcN or GlcNAc on mouse fibroblast proliferation. Experiments were carried out in 5 wells for each medium. The signal intensity of the proliferation of the cells on day 1 was determined by MTT assay. The results are expressed as the mean ± SD. Comparison of mean values was performed by Dunnett's *t*-testing. The statistical significance of the differences between cells cultured in a GlcN- or a GlcNAc-containing medium compared to cells cultured in a control medium was determined.

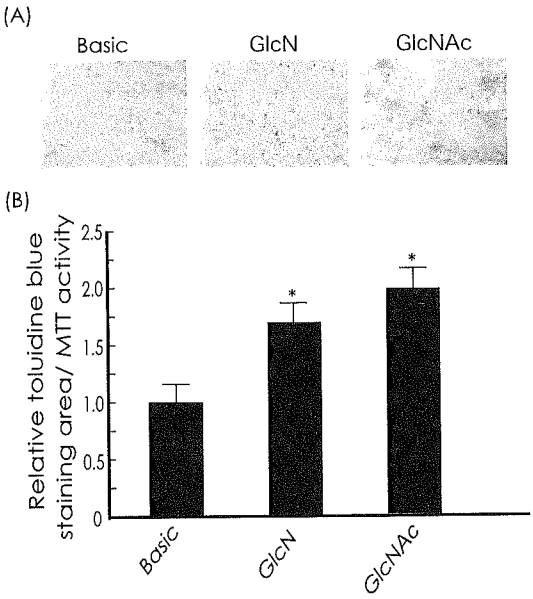

Fig. 4. Effect of GlcN or GlcNAc on GAG content and localization. (A) The impact of GlcN or GlcNAc on the toluidine-blue-stained area. Experiments were conducted in 5 wells for each medium. Each amino sugar was added to the basic medium at 5 mM. (B) Graph showing the ratio of the MTT assay to the signal intensity of the toluidine-blue-stained area when the cells were cultured with either GlcN or GlcNAc. The results are expressed as the mean ± SD. Comparison of mean values was performed by Dunnett' s *t*-testing. The statistical significance of the differences between cells cultured in a GlcN- or a GlcNAc-containing medium compared to cells cultured in a control medium was determined.

containing fibroblasts, keratinocytes, and melanocytes. By examination of the mouse fibroblasts analyzed in the *in vitro* component of the study, this component could examine whether GlcNAc supplementation had affected the amount of HA in the extracellular matrix (ECM) and increased *HAS2* and *RHAMM* mRNA expression levels. The investigation began with analysis of whether GlcN or GlcNAc supplementation affects the proliferation in the mouse fibroblast by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on day 2 of culturing (**Fig. 3**) to quantify fibroblast proliferation. The results indicated that whereas the metabolic activity of cells cultured in a GlcN-containing medium had decreased by approximately 0.75-fold compared to that of cells cultured in a basic medium ($p = 0.013$), the metabolic activity of cells cultured in a GlcNAc-containing medium had increased by approximately 1.35-fold ($p = 0.0002$). **Fig. 4 (A)** shows the results of toluidine blue staining, which was performed to identify the localization of GAG in GlcN-supplemented, GlcNAc-supplemented, or control cells cultured in the basic medium. As can be observed, a greater number of stained areas appear in the cells in the GlcN- and GlcNAc-containing media compared to the cells in the control medium.

Having found that GlcN and GlcNAc supplementation had affected fibroblast proliferation, densitometric analysis of the toluidine blue-stained areas was corrected by the value of MTT assay (**Fig. 4 (B)**). The results revealed that the GAG-staining area of fibroblast incubated in the GlcN- and GlcNAc-

containing media increased approximately 1.8- and 2.2-fold, respectively, compared with that of fibroblasts in the basic medium.

3.4 Has2 and RHAMM mRNA levels in the mouse fibroblast

Having that GlcN · HCl and GlcNAc supplementation had increased GAG content, the next step in the analysis was determining whether GlcNAc supplementation had affected gene expression in the fibroblast. After total RNA had been extracted either 4 h or 24 h after addition of GlcN or GlcNAc to the basic medium, the mRNA levels were measured by RT-PCR (**Fig. 5**). Compared to the cells cultured in the basic medium, the cells cultured in the GlcNAc-containing medium exhibited an approximately 2.2-fold increase in *HAS2* mRNA level at 4 h and an approximately 1.4-fold increase in *RHAMM* mRNA level at 24 h, an approximately 30% decrease in *HAS2* mRNA level at 24 h. Compared to the cells cultured in the basic medium, the cells cultured in the GlcN-containing medium exhibited a decrease in *HAS2* and *RHAMM* mRNA levels by approximately three-fourths at 4 h and a decrease in *HAS2* mRNA level by approximately two-thirds at 24 h but no change in *RHAMM* mRNA level at 24 h.

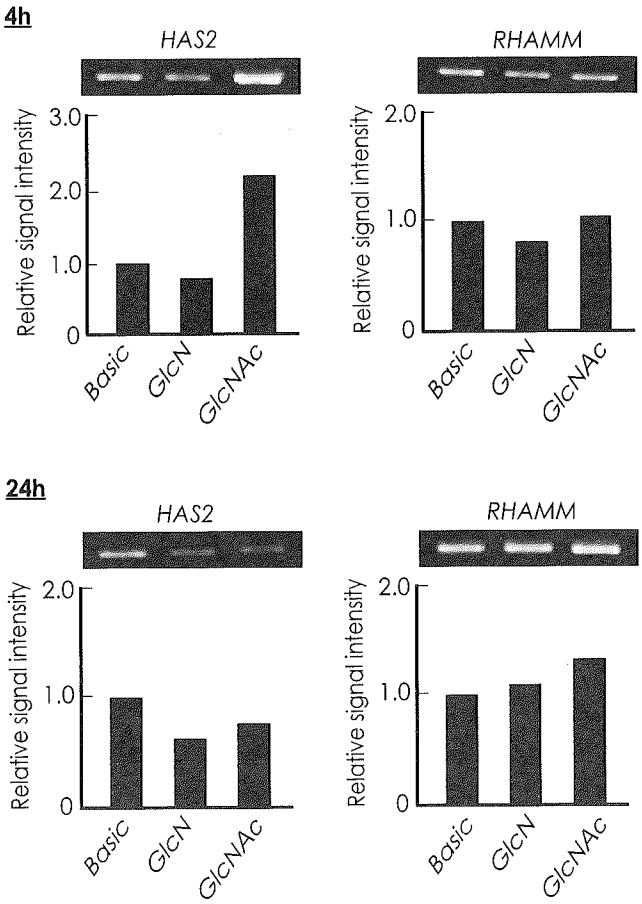

Fig. 5. Effect of GlcN or GlcNAc on hyaluronan synthase 2 (*HAS2*) and a receptor for hyaluronan-mediated motility (*RHAMM*) mRNA expression levels in ATDC5 cells. The results of RT-PCR analysis indicate that the *HAS2* and *RHAMM* mRNA levels were affected by GlcN or GlcNAc supplementation. The graph shows the band intensities determined using GelDoc software. The signals were normalized against those of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

4. Discussion

GlcN and GlcNAc are precursors of the production of HA, which plays a key role in maintaining moisture in dermal tissue. As GlcN or GlcNAc supplementation may supply the composition sugar of HA, it may increase the amount of moisture in the skin[8]. As such, GlcN or GlcNAc supplementation has been suggested to provide several cosmetic and health benefits, such as enhanced skin hydration and promotion of wound healing. Despite clinical observation of increases in skin moisture with GlcN or GlcNAc supplementation, there is little sound scientific evidence that it truly does so.

Recent studies have reported that GlcN and GlcNAc regulate the gene expression in endothelial cell, chondrocyte and osteoblast *in vitro* study[9-11].

However, these studies did not examine whether GlcN and GlcNAc regulate gene expression of skin tissue, nor did they investigate whether GlcN and GlcNAc, although similar molecularly, act in a different manner. To fill this research gap, this study examined whether GlcN and GlcNAc regulate gene expression in the skin in a manner that increases the amount of moisture retained in skin tissue and, if one or both indeed do so, whether they do so to a different extent and in a different manner. As can be observed in Fig. 1, which shows the results of gene expression profiling performed to determine the response of mouse skin to GlcN and GlcNAc supplementation, GlcNAc was found to regulate the gene expression of one about 3.5 times the number of this as compared with GlcN. As no differences in body weight and food consumption were observed among the 3 groups over the entire study period, nutritional factors, such as an energy intake, likely had no role in gene expression. Since the food consumption was about 3g/day/mouse, the mouse took in about 0.6 mg per day of Glc, GlcN·HCl, or GlcNAc (data not shown). The results thus indicate that oral ingestion of GlcN and GlcNAc had changed the gene expression of mouse dermal tissue via digestion and absorption, as well as that each did so to a different extent.

The content of HA in tissue is regulated by the synthesis of HA by HAS located on the inner surface of plasma membrane and its subsequent catabolism[12-16]. To date, 3 isoenzymes of *HAS*—*HAS1, HAS2,* and *HAS3*—have been detected in humans and mice. Human dermal fibroblasts abundantly express *HAS1* and *HAS2*, whereas epidermal keratinocytes predominantly express *HAS3*[17, 18]. The results of gene cluster analysis performed to examine the increase in mRNA expression levels with GlcNAc supplementation indicated an interaction among 8 genes related to HA metabolism or an HA pathway. First, *HAS2* was found to have been upregulated by approximately 4.3-fold in the GlcNAc-supplemented mice compared to the control mice (Fig. 2), indicating that GlcNAc supplementation may have accelerated HA production in the mice. In this experiment, RNA samples isolated from skin tissue containing epidermal keratinoxytes and dermal fibroblasts were used. As GlcNAc supplementation was found to have upregulated the mRNA expression level of *HAS2*, it is hypothesized that GlcNAc may regulate the HA production by affecting the expression of HAS2 in fibroblasts.

Degradation of HA is mediated by the HA-degrading enzyme *HYAL*. Of the 6 *HYAL* genes that have been identified, only 3—*HYAL1, HYAL2,* and *HYAL3*—are ubiquitously expressed in somatic tissue[19]. HYAL2 is a lysosomal hyaluronidase that specifically hydrolyzes HA into 20-kDa fragments[19, 20]. The finding that GlcNAc supplementation resulted in an approximately 3.0- and 2.6- fold upregulation on 2 points of the *HYAL2* gene (Fig. 2) indicates that GlcNAc supplementation might promote not only HA synthesis but HA degradation, and thereby accelerate HA metabolism.

Dermal skin contains various kinds of proteoglycans, including heparan sulfate, chondroitin sulfate, and dermatan sulfate[21-26]. The existence of the HA and proteoglycan binding link protein gene family (*Hapln*), whose 4 members are physically linked adjacent to chondroitin sulfate proteoglycan core protein genes, and the expression profile of its members suggest that HA–proteoglycan aggregates are present in most vertebrate tissues[27]. HA may be found in variable amounts in many connective tissues, where it is usually bound by large aggregating chondroitin sulfate proteoglycans, such as versican and aggrecan, via the Hapln or cartilage link protein[28]. The composition of each aggregate in dermal tissue remains unclear, but it is hypothesized that each Hapln protein may be able to bind selectively to specific proteoglycans and to stabilize the resultant aggregates. In this study, the genes of several proteins associated with HA were found to have been upregulated by GlcNAc supplementation, including *Hapln2* and *Hapln4,* which were found to have been upregulated by

approximately 2.4- and 2.0-fold, respectively. In addition, *HABP4*, a member of a gene family (*HABP*) whose members might act as cell-surface binding sites for HA[29], was found to have been upregulated by approximately 3.0-fold with GlcNAc supplementation. These results indicate that GlcNAc supplementation might change the environment of extracellular matrices, including those composed of HA.

Many of the effects exerted by HA are mediated through cell-surface receptors, of which 5 have been molecularly characterized, namely *CD44, RHAMM, Intercellular adhesion molecule 1* (*ICAM-1*), *Layilin, and Lymphatic vessel endothelial hyaluronan receptor 1* (*Lyve1*). Binding of the HA ligand to its receptors triggers signal-transduction events, which, in concert with other ECM and cytoskeletal components, can direct cell trafficking during physiological and pathological events[30]. In this study, the *RHAMM*, *CD44, ICAM1, Layilin*, and *Lyve1* expression levels of GlcNAc-supplemented mice were found to have been upregulated by approximately 2.1- (**Fig. 2**), 0.5-, 1.0-, 0.4-, and 2.5-fold (data not shown), respectively, compared to control mice (data not shown). Moreover, GlcNAc supplementation was found to have upregulated the expression of *TGFb1*, a growth factor that stimulates HA synthesis[31-33], by approximately 8.6-fold (**Fig. 2**), indicating that GlcNAc supplementation had accelerated HA metabolism by increasing *TGFb1* expression.

In the *in vivo* component of this study, a mixture of skin tissues consisting of fibroblasts, keratinocytes, and melanocytes was examined. The DNA microarray data revealed that GlcNAc supplementation had increased *HAS2* expression and its upstream and downstream genes in mouse skin tissue. Hence, we examined whether GlcNAc affect the amount of HA in extracellular matrix and raised *HAS2* and *RHAMM* mRNA expression levels using by fibroblast of mouse *in vitro* study. The results revealed that GlcNAc supplementation had increased the GAG staining area and increased *HAS2* and *RHAMM* mRNA levels by approximately 2.2- and 1.4-fold, respectively. These findings indicate that GlcNAc might promote HA synthesis and production, which in turn upreglates the mRNA expression of *RHAMM*, the HA receptor. The finding of *in vitro* study using fibroblasts indicate that GlcNAc supplementation affects mouse dermal tissue by altering the ECM metabolism relevant to HA via the mRNA expression of fibroblasts. As GlcN was not found to affect the mRNA expression levels of *HAS2*, *RHAMM*, *HYAL2*, *Hapln2*, *Hapln4*, or *TGFb1* but was found to increase the staining area of GAG in the ECM of the mouse fibroblast, it is hypothesized that GlcN might regulate GAG metabolism using a different mechanism than does GlcNAc.

In conclusion, the results of this study indicate that intake of GlcNAc might accelerate HA metabolism via the upregulation of mRNA expression of *HAS2*, *Hyal2* and *RAHMM*. By providing an understanding of the mechanism by which GlcNAc acts on fibroblasts, this study assists in providing a scientific basis for the efficacy of GlcNAc supplementation in increasing the HA content of dermal tissue as a means of increasing skin moisture. Further research is necessary to investigate the efficacy of GlcNAc supplementation in special applications targeting cosmetic enhancement and wound healing.

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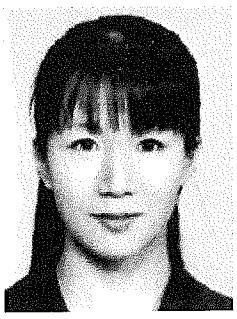

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